Author's response to reviews

Title: Differential Diagnosis of Tuberculous meningitis from partially treated cases of pyogenic meningitis by Cell ELISA.

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Author's response to reviews: see over
Differential Diagnosis of Tuberculous Meningitis from partially treated cases of pyogenic meningitis by Cell ELISA.


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Running title: Diagnosis of tuberculous meningitis
Abstract

**Background:** Tuberculous meningitis (TBM) is a major global health problem wherein the difficulty in diagnosis gets more complicated with partially treated cases such as pyogenic meningitis (PTPM). In our earlier study we have demonstrated the presence of a 30 kD protein antigen in cerebrospinal fluid (CSF) of TBM patients. We have used this protein to show that lymphocytes derived from CSF respond differentially in TBM with that of derived from PTPM patients.

**Methods:** - A cell enzyme linked Immunosorbant assay (Cell ELISA) has been developed for the quantitative measurement of anti-30 Kd protein antibody production by B cells from CSF of TBM and PTPM patients.

**Objective:** - Our study is intended to develop an assay, which can discriminate the TBM cases from the cases of PTPM. The methodology used also should be easily adaptable for pathology laboratory.

**Results:** - The assay gave 92% (11/12) sensitivity and 92% (11/12) specificity for the differential diagnosis of TBM from PTPM.

**Conclusion:** - The B cells derived from CSF of TBM patients respond to IgG production within 24h when they are induced with 30 kD protein antigen for *Mycobacterium* but it was not been observed for B cells derived from PTPM patients.
**Keywords:** 30 kD protein antigen, tuberculous meningitis, partially treated cases of pyogenic meningitis, Cell ELISA.

**Running title:** - Diagnosis of TBM

**Background**

Tuberculous meningitis (TBM) is the infection of the Central Nervous System (CNS) and is prevalent in under-developed and developing countries. There is resurgence of TBM due to the growing number of people infected with Human Immunodeficiency Virus (HIV). Diagnosis of TBM remains a problem despite many new and advanced methodologies [1,2]. Previous clinical studies of TBM have clearly demonstrated that the timing of treatment is the most critical factor affecting the ultimate outcome, which stresses the importance of early diagnosis of TBM [3]. The laboratory confirmation for the diagnosis of TBM is based on the detection of acid-fast bacilli (AFB) in the cerebrospinal fluid (CSF) and by culturing CSF for Mycobacterium tuberculosis bacilli (MTB) [4]. The sensitivity of direct AFB smear in the CSF is only 5-10% and culturing techniques take 4-6 weeks. It has been recently reported that the staining efficiency of AFB smear test can be increased up to 50% % of TBM cases, but a very large amount of CSF sample is required [5].
The clinical as well as CSF features of TBM are helpful indicators for the diagnosis of TBM and can not differentiate with other infectious and non-infectious disorders [6-7] but clinicians often have difficulty in differential diagnosis of TBM from partially treated cases of pyogenic meningitis (PTPM). The biochemical and pathological analysis of CSF and clinical picture in TBM can often be similar to that of PTPM which results in frequent diagnostic confusion.

In an earlier study, we have reported the presence of a diagnostic 30 kD protein antigen in CSF of confirmed and suspected cases of TBM patients [8]. Immunological methods such as antibody capture ELISA have been devised for the diagnosis of TBM [9]. The cell ELISA method developed gives further confirmation of the results obtained by antibody capture ELISA.

Cellular immune function is characterized by the existence of various types of lymphoid cells. Lymphocytes take part in the production of humoral mediated immunity and therefore it is of interest to study these cells in response to the 30 kD protein antigen in TBM and PTPM cases. Cell Enzyme Linked Immunosorbant assay (Cell ELISA) method has been developed to study the response of B cells derived from CSF of TBM and PTPM cases by challenging with 30kD protein antigen. It is of interest to see
whether this method is useful to avoid diagnostic confusion between TBM and PTPM patients. By using Cell ELISA the above problem might be circumvented and sensitivity and specificity might be enhanced by evaluating the antibody response at a cellular level. The present study evaluates the antibody response to 30 kD protein antigen in CSF of TBM/PTPM patients by Cell ELISA.

**Subjects and Methods**

**Hospital setting, Patients and collection of samples.**

Central India Institute of Medical Sciences (CIIMS), Nagpur is a tertiary referral center. Study on the CSF of patients suspected of TBM or other infections were collected before starting the treatment. However in patients of cranial surgery, CSF examination is done when they were suspected to have developed meningitis. These patients were already on broad spectrum antibiotics like third generation cephalosporins, aminoglycosides etc.

For establishing the diagnosis of meningitis, 2-5 ml CSF was withdrawn from all these patients by Lumbar puncture. CSF was subjected to routine biochemical analysis and pathological analysis including Gram, India ink, Acid Fast Bacilli staining and culturing. One ml of CSF was used for the cell ELISA study. In addition part of the CSF was used for detection of 30 kD protein band by SDS-
PAGE. Diagnosis of TBM and partially treated pyogenic meningitis was based on the criteria given below:

**Diagnostic Criteria**

1. **Tuberculous Meningitis (TBM):** Presence of Mycobacterium tuberculosis in CSF by staining and/or culture. OR Clinical meningitis with following observations
   A) Sub-acute or chronic fever with features of meningeal irritation like headache, neck stiffness, vomiting with or without other features of CNS involvement
   B) CSF findings showing increased proteins, decreased glucose (CSF: Blood glucose ratio less than 0.5), Pleocytosis with lymphocytic predominance.
   C) Presence of 30 Kd protein band in CSF on SDS-PAGE.
   D) Good clinical response to antituberculous drugs.

None of the 12 patients of TBM included had shown AFB on staining.

2. **Partially treated pyogenic meningitis (PTPM)** Presence of pathogenic bacteria in CSF by staining and/or culture. OR Clinical meningitis with following observations
   1) Fever, signs of meningeal irritations in post cranial surgery cases of tumors, strokes, head injuries etc receiving antibiotics OR High fever, signs of meningeal irritations with or without CNS manifestations receiving broad spectrum antibiotics.
2) CSF finding showing: Increased proteins, decrease glucose (CSF: Blood glucose ratio less than 0.2) Pleocytosis with predominance of polymorphonuclear cells. At times CSF picture resembles that of chronic meningitis.

3) Absence of 30Kd protein band in CSF on SDS-PAGE.

4) Good clinical response to broad-spectrum antibiotics

From these two groups (TBM/PTPM) 1ml of CSF from each of the 12 patients selected randomly and was used for cell ELISA.

3. **Control group**: The peripheral blood samples from six healthy volunteers were also analyzed and included as negative control.

**Laboratory studies:**

**Sodium dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):**

CSF samples obtained from confirmed and suspected cases of TBM were subjected to SDS-PAGE. SDS-PAGE was performed with vertical slab gel electrophoresis system (BROVIGA, INDIA) by using standard Laemmali method (10). 4% stacking gel and 10% running gel were used. The electrophoresis was carried out at 250 volts at 50 mAmperes. The gels were developed by staining with coomassie brilliant blue GR-250 and the protein
profiles were studied. The band size (molecular weight) estimated using molecular weight markers (Genei, Bangalore, India) in the a parallel lanes.

**Preparation of antigen (30kD):** After the separation of protein from CSF of confirmed TBM patient (AFB Positive) by SDS-PAGE, the region where 30 kD protein band observed was sliced from the gel and preequilibrated in the elution buffer (0.15M PBS, pH-7.4) and then the gel (containing 30 kD protein antigen) was electro eluted in a whole gel eluter system (BIOTECH, INDIA) for 90min, at 30 volts (11), and harvested from the unit and dialyzed against PBS and the protein content was measured by Bio Lab KIT. The purity of protein was checked by running Native PAGE and was used in the study for the evaluation of antibody response of B cells derived from CSF of TBM and PTPM cases.

**Preparation of CSF Cells:** One ml of CSF sample collected from both types of patients (TBM/PTPM) was centrifuged at 400 rpm for about 20 min. The supernatant was discarded and cell pellet was washed twice with PBS and then diluted in RPMI 11640 tissue culture medium containing 10% of fetal calf serum.

**Preparation of Blood Cells:** - Heparinized blood samples were obtained from six healthy volunteers. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by standard Ficoll-Hypaque gradient
centrifugation. The PBMC were dissolved in PBS and centrifuged at 400 rpm for about 15-20 min, PBMC were diluted in RPMI 11640 tissue culture medium containing 10% of fetal calf Serum.

**Cell ELISA:** 96 well ELISA plates with a flat bottom were coated with 10ugm of 30kD /ml diluted in phosphate buffer saline (PBS) pH 7.2.After overnight incubation the plates were washed with PBS and then coated with 5% BSA-PBS for 4 hrs. The plates were again washed five times with the PBS.200 ul of the cell preparation derived from CSF of patients with TBM and PTPM was then added in the wells and coded. Each sample was prepared in duplicate. Plates were kept overnight at 37°C in 5% CO₂ in a carbon dioxide incubator. Next day plates were washed with PBS and Horseradish peroxides (HRPO) conjugated rabbit anti human IgG (1:10,000) was then added to the plate. After 2 hrs of incubation at 37°C the plates were washed again with the PBS and 100 ul of TMB/H₂O₂ was added. The TMB/H₂O₂ act as substrate for the HRPO enzyme. After 15 minutes of incubation 100 µl of stop solution (2.5 N Sulphuric acid) was added and the plates were then read with an ELISA reader at 450nm (12).

**Results**

The detailed clinical data of TBM and PTPM patients is presented in Table-1. Out of the 12 patients of PTPM, microorganisms were observed in 2
cases and cultured (gm+ve cocci in one case and gm-ve bacilli in the another case). Among the 12 patients who fulfilled the criteria for TBM as shown in table –1, CSF was positive for the 30 kD protein antigen but did not show AFB on staining. None of the patients had given a previous history of extra CNS tuberculosis. We have tested more than 700 CSF samples including 150 TBM patients. In more than 90% of the TBM patients the 30 kD protein antigen was observed (Data not shown). Figure 1 shows the presence of 30 kD protein band in the CSF of suspected cases of TBM. This band was markedly absent in CSF of the PTPM patients.

The ELISA absorbance values of IgG to 30 kD protein antigen in CSF of TBM and PTPM patients are presented in Figure 2. The cut off values (O.D at 450nm) for positivity to 30 kD protein antigen IgG in the control CSF is 0.6. Out of 12 patients of TBM, high titer values of IgG antibody production against 30kD protein antigen were observed in 11 cases. However, in PTPM patients the titer was very low as compared to the cases of TBM patients. IgG antibody production expressed as ELISA absorbance value ranged from 0.7 to 2.0 for cells derived from CSF of TBM patients (Except case no.5 {0.59}) and from 0.05 to 0.38 for cells derived from CSF of pyogenic meningitis cases (Except case no.4 {0.79}). The cell ELISA gave sensitivity of 92% and specificity of 92% for differential diagnosis of TBM from
PTPM. No IgG antibody production to 30 kD protein antigen was produced by peripheral blood lymphocytes from 6 healthy individuals within 48 hrs.

**Discussion**

During the past decade, several conventional immunoassays such as ELISA, Dot Immunobinding assay, immunoblot assay, and various molecular methods such as Polymerase chain reaction (PCR) have been reported as adjuncts in the diagnosis of TBM [4,13,14,15]. However many of the aforementioned techniques have been reported to have problems in differentiating TBM from PTPM. CSF TLC, DLC, protein and glucose estimation are helpful parameters for diagnosis of TBM and for differentiating other infectious and non-infectious neurological disorders but are non-specific and often cannot differentiate from PTPM where organisms are often not seen and grown. Delay in diagnosis and treatment are regarded as major contributing factors in the high mortality and morbidity. Delay in starting appropriate medication for TBM and PTPM worsens outcome.

We have demonstrated by SDS-PAGE a 30kD protein antigen marker in CSF of suggests that the selected protein band (marker) carries the candidate protein marker antigen, which is specific to *M.tuberculosis* and could be considered as diagnostic marker for TBM. Therefore 30 kD protein antigen
was chosen as the antigen for evaluation of the IgG antibody response of B cells derived from CSF of TBM, PTPM and blood samples from six healthy volunteers. A Cell ELISA was developed for the quantitative measurement of antibody production against 30kD protein antigen by these cells. High titers of IgG antibody production were observed in TBM as compared to the cases of PTPM patients. The cells obtained from CSF of TBM patients give an early response since they are already sensitized against the MTB antigens. However the cells obtained from PTPM patients and healthy volunteers when challenged with the 30 kD protein antigen give a delayed response since they are not sensitized against this antigen. Therefore on time scale an early response indicates TBM.

The Cell ELISA has been shown to be a sensitive technique for the differential diagnosis of TBM from PTPM. This method involves the demonstration of active antibody production by cells, particularly those derived from the affected site [16]. Earlier in our laboratory we have standardized Cell ELISA methodology using standard culture filtrate protein of M.tuberculosis of H37Rv strain received from Colorado State University U.S.A (Data not shown). The only limitation of this study is the time period involved; 24-30 hours. However, the sensitivity of the test overcomes this
drawback since it the only reported method that can discriminate TBM from PTPM.

The sensitivity and specificity of IgG antibody for the differential diagnosis of TBM from PTPM using 30 kD protein antigen by Cell ELISA was found to be 92%(11/12). We have demonstrated that antibody production against 30 kD protein antigen is higher in the cells derived from CSF of patients with TBM than in those from PTPM.

Various methods were developed in our laboratory for the diagnosis of TBM showing high specificity and sensitivity but in few cases false positive results were observed in cases of pyogenic meningitis especially in PTPM [17,18]. The Cell ELISA method developed in our laboratory using 30 kD protein antigen marker may be useful in such circumstances and can give additional information to the treating physician to differentiate TBM cases from PTPM cases.

The Cell ELISA method for the diagnosis of TBM is based on the assumption of local synthesis of humoral antibody against MTB antigen. Various workers showed that CSF derived cells had a significantly higher proliferate response to purified protein derivative (PPD) in patients with TBM suggesting an intrathecal immune response [11,19].
In summary, the data of present study highlights three important relevant observations. Firstly, Cell ELISA is useful method for differentiating TBM from PTPM using 30 kD protein antigens; secondly the method of challenging the B-lymphocytes from the CSF of suspected TBM with 30 kD protein antigen can be helpful in confirming the diagnosis of TBM and thirdly the assay allows several samples to be analyzed simultaneously. Hence the proposed assay may be very useful tool in differential diagnosis of TBM from PTPM.

**Conclusion**

The presence of 30 kD protein antigen in CSF of TBM patients indicates that this protein carries the candidate marker antigen which is specific to *M.tuberculosis*. We have demonstrated that by using Cell ELISA method we can differentiate TBM from PTPM patients which will be helpful for diagnosis of TBM. In addition, our data provides confirmation of the observations about the immune response of CSF cells.

**Acknowledgement**
We would like to acknowledge the help of Colorado State University, USA and NIH, NIAID Contract No.1 AI-75320, entitled ‘Tuberculosis Research Materials and Vaccine Testing’

**Competing Interest statement**

The authors declare that they have no competing financial interests.

**Authors’ contribution**

RSK carried out study design, data collection, statistical analysis, data interpretation, literature search, and manuscript preparation, NPA, RPK, and RMS assisted in data analysis collection, NHC assisted in data collection, statistical analysis, data interpretation, HJP participated in the preparation of manuscript, data interpretation and study design, GMT provided his assistance in preparation of manuscript, data interpretation, study design and funds collection and HFD supervised the study design, statistical analysis, data interpretation manuscript preparation and literature search.

**References:**


### TABLE-1. Presentation of Clinical & CSF findings of Tuberculous & Partially treated pyogenic meningitis patients

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/Sex</th>
<th>TLC Cu.mm</th>
<th>%P</th>
<th>%L</th>
<th>Protein (mg/dl)</th>
<th>Sugar (mg/dl)</th>
<th>CSF: blood Sugar ratio</th>
<th>Neck Stiffness</th>
<th>Duration of fever (weeks)</th>
<th>Headache</th>
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<tr>
<td>1*</td>
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Chest X ray = *Pulmonary Tuberculosis   ** Consolidation

**PTPM:** Case 1, 2, 3, 5, 8, 10, 11 = Cranial surgery post operative. Case 7, 9 = Post Head injury. Case 4, 6, 12 =

Presented to CIIMS as PTPM.

+ ------ Few gm-ve bacilli seen, ++++ -- Few gram+ve cocci in pairs (non capsulated)
Figure-1. SDS-PAGElectrophoretogram of CSF from control (lanes B, C, E) and suspected TBM subject (lane D) along with Molecular weight marker (lane A). The arrow indicates the 30 Kd band (30 Kd protein antigen).
Figure 2. B Cell response (IgG reactivity) to 30 KD protein antigen in CSF cells derived from (Tuberculous meningitis) TBM and partially treated pyogenic meningitis (PTPM) and blood cells from control subjects.